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Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin $\alpha_{IIb}\beta_3$

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Blood loss at sites of vascular rupture is controlled by the adhesion and aggregation of platelets and the formation of an insoluble fibrin matrix. Fibrinogen is considered to be critical in these processes by both providing an abundant dimeric ligand for $\alpha_{IIb}\beta_3$-mediated platelet aggregation, and serving as the fundamental building block of the fibrin polymer. To establish an *in vivo* model system to examine in detail the importance of $\alpha_{IIb}\beta_3$–fibrinogen interactions in platelet function, hemostasis, response to injury and vaso-occlusive disease, and to test the prevailing hypothesis that the C-terminal segment of the fibrinogen γ chain is essential for $\alpha_{IIb}\beta_3$ binding, we have used gene-targeting technology in mice to eliminate the last five residues (QAGDV) from the γ chain. Mice homozygous for the modified γ chain gene (γΔ5/γΔ5) displayed a generally normal hematological profile, including normal platelet count, plasma fibrinogen level, clotting time and fibrin crosslinking. However, both γΔ5–fibrinogen binding to $\alpha_{IIb}\beta_3$ and platelet aggregation were highly defective. Remarkably, another $\alpha_{IIb}\beta_3$-dependent process, clot retraction, was unaffected by the γΔ5 mutation. Despite the preservation of clotting function, γΔ5/γΔ5 mice were unable to control blood loss following a surgical challenge and occasionally developed fatal neonatal bleeding events.

Keywords: adhesion/fibrinogen/gene targeting/integrin/platelet

Introduction

Vascular integrity is preserved by a sophisticated system of circulating and cell-associated hemostatic factors that control both the local deposition of platelets and the conversion of soluble fibrinogen to an insoluble fibrin matrix (Doolittle, 1994; Lind, 1995). Platelets play a critical role in the initial restriction of blood loss following vascular injury by rapidly adhering to exposed subendothelial matrix components and aggregating to form a provisional plug. The extraordinary adherence properties of platelets that are pivotal to their function in hemostasis are conferred by a wide array of receptors for specific extracellular ligands and matrix components, including fibrin(ogen), von Willebrand factor (vWF), collagen, fibronectin and vitronectin (Charo *et al.*, 1994; Ruggeri, 1995; Sixma *et al.*, 1995). These receptors provide a means to adhere at essentially any site of injury regardless of the local shear stresses imposed by blood flow or the matrix composition. A particularly abundant and clinically important platelet adhesion molecule is the integrin, $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) (George *et al.*, 1990; Calvete, 1994; Caen and Rosa, 1995). The significance of this platelet-specific integrin in hemostasis and platelet function is emphasized by the fact that patients with severe forms of Glanzmann's thrombasthenia, which lack $\alpha_{IIb}\beta_3$, often suffer sustained bleeding events and exhibit profound defects in both platelet aggregation and clot retraction (George *et al.*, 1990; Caen and Rosa, 1995). The clinical significance of $\alpha_{IIb}\beta_3$ is further highlighted by the fact that platelet aggregation, mediated by this receptor, may be primarily responsible for life-threatening re-occlusion events in coronary vessels initially cleared of an infarct by the application of thrombolytic therapy or surgical intervention.

The biological linkage between $\alpha_{IIb}\beta_3$ and platelet adhesion and aggregation can be at least partially understood based on the highly regulated binding activity of this integrin and its ligand specificity. In order for $\alpha_{IIb}\beta_3$ to bind soluble ligands and most immobilized ligands, the platelets must be activated with agonists such as ADP or thrombin. Signal transduction systems mediating platelet activation appear to trigger a conformational alteration in the extracellular domain of $\alpha_{IIb}\beta_3$, changing it from a low to a high affinity receptor (O'Toole *et al.*, 1990; Sims *et al.*, 1991; Ginsberg *et al.*, 1995). A key ligand for the 'activated' receptor is soluble fibrinogen (Fbg), a dimeric protein ($A\alpha_2B\beta_2\gamma_2$) carrying at least two sites of interaction with $\alpha_{IIb}\beta_3$. Once activated, platelets rapidly become linked into aggregates using Fbg as a bridging molecule. The subsequent coupling of extracellular fibrin(ogen) to cytoskeletal contractile proteins through $\alpha_{IIb}\beta_3$ appears to drive the process of clot retraction (Bertagnolli and Beckerle, 1993; Schoenwaelder *et al.*, 1994).

Other ligands are also known to interact with $\alpha_{IIb}\beta_3$, including vWF (Ruggeri *et al.*, 1983), fibronectin (Plow *et al.*, 1985b) and vitronectin (Pytela *et al.*, 1986), and these may also serve important roles in platelet adhesion, aggregation and spreading. Studies of thrombus formation *in vitro* under flow conditions strongly suggest that vWF binding to GPIbα and $\alpha_{IIb}\beta_3$ may be particularly important for platelet adhesion and aggregation at high shear rates (Savage *et al.*, 1996). One fundamental question raised by the extraordinary number and diversity of ligands recognized by $\alpha_{IIb}\beta_3$, is what precisely constitutes the ligand binding motif of this integrin? This information is fundamental to understanding the molecular events that lead to the induction of $\alpha_{IIb}\beta_3$ binding activity in activated platelets, the molecular basis for $\alpha_{IIb}\beta_3$ binding specificity and the secondary events triggered by ligand binding

(Shattil *et al.*, 1994). Definition of the ligand binding motif might also prove to be highly instructive in the development of effective clinical strategies to treat platelet-associated vaso-occlusive events such as myocardial infarction and stroke.

A substantial body of work points to RGD sequences as a component of the structure recognized by $\alpha_{IIb}\beta_3$. Mutational analyses have shown that RGD sequences within vWF (Beacham *et al.*, 1992) and vitronectin (Cherny *et al.*, 1993) are essential for binding to $\alpha_{IIb}\beta_3$. Furthermore, a variety of RGD-containing synthetic peptides have been shown to block the binding of all ligands to $\alpha_{IIb}\beta_3$ and effectively inhibit both platelet aggregation and clot retraction *in vitro* (Gartner and Bennett, 1985, Haverstick *et al.*, 1985, Plow *et al.*, 1985a, 1987; Gartner and Ogilvie, 1988; Hantgan, 1988). Human Fbg has two RGD sequences, one in the coiled-coil portion of the Aα chain (α95–97) and one in the C-terminal portion of the Aα chain (α572–574); short peptides encompassing these sequences are effective inhibitors of $\alpha_{IIb}\beta_3$. However, the importance of the Fbg RGD sequences in binding $\alpha_{IIb}\beta_3$ has been frequently questioned, based on the lack of conservation of these sequences between species (Murakawa *et al.*, 1993), the binding activity of proteolytic fragments of Fbg lacking RGD sequences (Savage and Ruggeri, 1991), the inability of RGD-directed antibodies to suppress binding activity (Cheresh *et al.*, 1989) and the ability of non-RGD peptides derived from the C-terminal portion of the γ chain to inhibit $\alpha_{IIb}\beta_3$ and block platelet aggregation (Kloczewiak *et al.*, 1984). Furthermore, mutagenesis studies of recombinant human Fbg expressed *in vitro* showed that the RGD sequences were neither necessary nor sufficient for platelet aggregation (Farrell and Thiagarajan, 1994), whereas the final C-terminal residues of the γ chain appeared to play a role (Farrell and Thiagarajan, 1994; Rooney *et al.*, 1996).

To develop an *in vivo* model system to explore in detail the role of $\alpha_{IIb}\beta_3$–Fbg interaction in platelet function, hemostasis, response to injury and the pathobiology of vaso-occlusive diseases, and to test definitively the hypothesis that the five C-terminal residues of the γ chain are a crucial component of the platelet receptor binding motif of Fbg, we have used gene-targeting technology to selectively modify the endogenous γ chain gene in mice. We report that mice expressing a form of the γ chain lacking five amino acid residues display a generally normal hematological profile, including normal platelet count, plasma Fbg level, clotting function and factor XIII-mediated γ chain crosslinking. However, the mutant Fbg was highly defective in both $\alpha_{IIb}\beta_3$ binding and platelet aggregation. Consequently, these mice were unable to control blood loss following vascular injury and sustained fatal bleeding events as neonates, but with low penetrance.

Results

Truncation of the fibrinogen γ chain in mice

The endogenous γ chain gene was modified in mouse embryonic stem (ES) cells to encode a truncated polypeptide using a replacement-type targeting vector prepared from the cloned mouse γ chain gene (Figure 1A). The specific nucleotide substitutions introduced into the targeting vector (Figure 1A and B) resulted in the conversion of the last codon of exon 9 (Gln407) into a translation termination codon. The selection of this codon for site-directed mutagenesis was not arbitrary. This constitutes the only codon change that would: (i) encode the same truncated polypeptide product regardless of whether the splicing and polyadenylation of γ chain mRNA followed the γ or γ′ pattern (see below), and (ii) preserve the residues in the γ chain that participate in transglutaminase (factor XIII) crosslinking of fibrin (see Figure 1B). It is important to note that in both humans (Chung and Davie, 1981) and rodents (Crabtree and Kant, 1982), the last two exons in the γ chain gene are subject to alternative splicing/polyadenylation. The majority of circulating Fbg is assembled with a form of the γ chain carrying the four C-terminal residues encoded by exon 10, but a small fraction (~10%) is assembled as a heterodimer with a variant of the γ chain of uncertain function, termed γ′. This variant is generated by a failure to splice exons 9 and 10, and results in the replacement of the last four amino acids by an entirely different sequence (Chung and Davie, 1981; Crabtree and Kant, 1982, and see Figure 1B). Thus, a simplifying feature of the selected γ chain gene mutation is that the encoded translation product is rendered insensitive to the splicing of exons 9 and 10. Furthermore, the mutant protein, termed γΔ5, is just five amino acids shorter than the standard form of the γ chain (Figure 1B).

Of 235 ES cell clones examined that had stably incorporated the transfected targeting vector, 28 were found to have incorporated the transgene by homologous recombination, based on PCR and Southern blot analysis (data not shown). Sequence analyses of the PCR products generated from 10 positive clones confirmed that six had the desired termination codon in exon 9 (note that only a portion of the legitimately targeted ES cell clones will carry the termination codon in exon 9 given an arbitrary recombination crossover point between the endogenous gene and the long arm of the targeting vector). Three of these were injected into C57Bl/6 blastocysts to generate chimeric transgenic mice. Eight of 20 chimeric males generated subsequently transmitted the modified γ chain gene to their offspring. Interbreeding of heterozygous mice (wt/γΔ5) [wild-type and targeted γ chain alleles are designated wt and γΔ5, respectively] resulted in the birth of γΔ5/γΔ5 offspring based on both PCR (data not shown) and Southern blot analyses (see representative data in Figure 1C). The distribution of genotypes among 100 progeny sampled from heterozygous parents was 27 wt/wt, 47 wt/γΔ5 and 26 γΔ5/γΔ5, indicating that neither wt/γΔ5 nor γΔ5/γΔ5 offspring were selectively lost during development.

γΔ5/γΔ5 mice express normal levels of fibrinogen Aα, Bβ and γ chain mRNA and normal levels of plasma fibrinogen

The nucleotide substitutions introduced into the γΔ5 allele placed no obvious constraints on γ chain mRNA splicing or polyadenylation, therefore, the expression of the mutant allele was not expected to be impaired. Nevertheless, to verify that the modifications of the Fbg γ chain gene did not compromise the normal hepatic expression of any of the three Fbg polypeptide chains, the Aα, Bβ and γ chain mRNA levels were examined in total RNA extracts prepared from the livers of 8-week-old wt/wt, wt/γΔ5 and

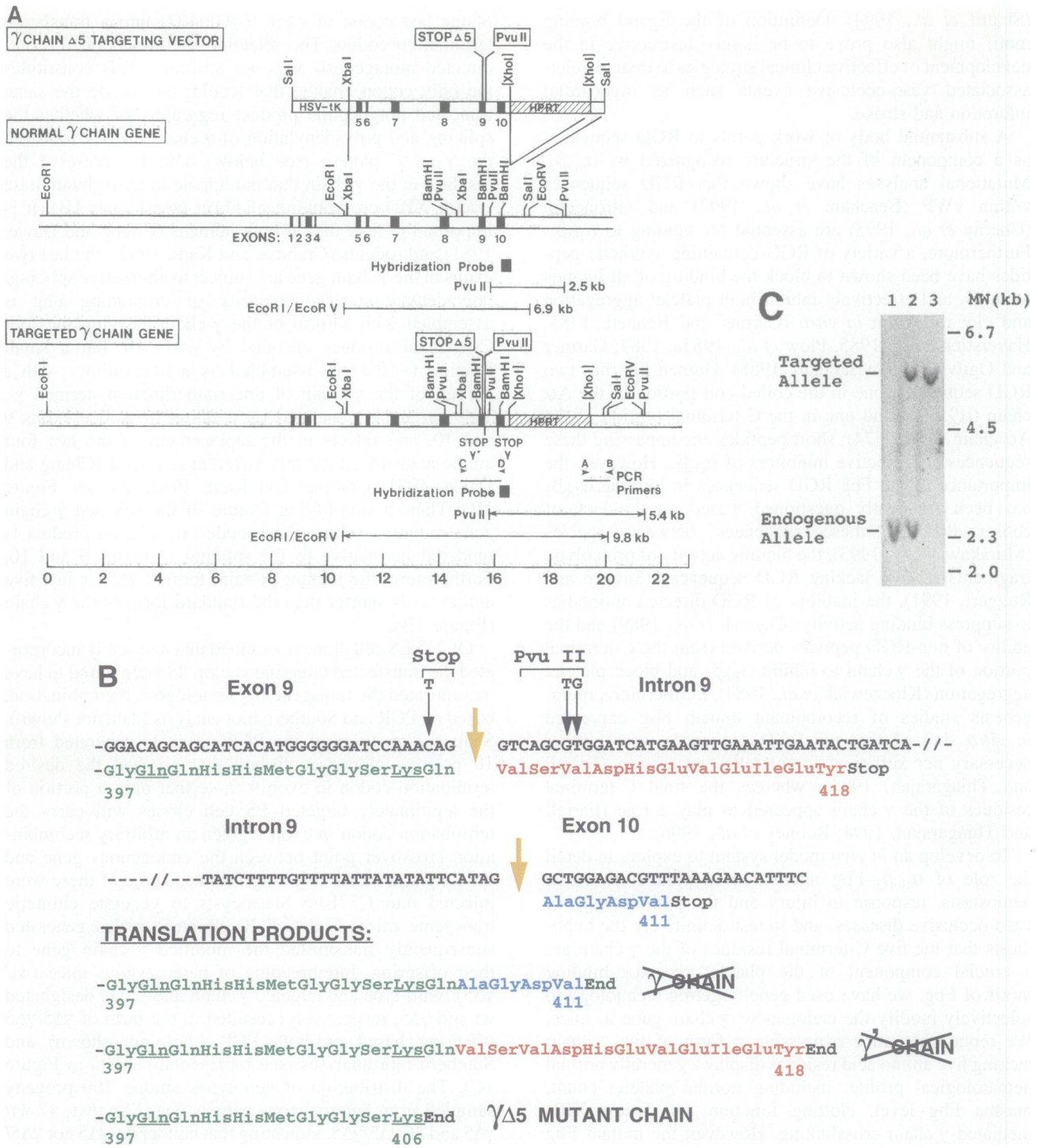

Fig. 1. Targeting of the Fbg γ chain gene by homologous recombination. (**A**) Structure of the Fbg γ chain targeting vector, the normal mouse Fbg γ gene and the targeted Fbg γ gene. Solid black boxes indicate location of exons and open boxes indicate intron sequences and 3′-untranslated sequences. The HPRT and HSV-tk minigenes were introduced into the targeting vector as selectable markers. The locations of the translation termination codon introduced into exon 9 and the *Pvu*II site introduced into intron 9 are box-labeled as Stop Δ5 and *Pvu*II, respectively. Solid box labeled as Hybridization Probe indicates the region complementary to the probe used for genomic Southern blot analysis. Positions and sizes of *Pvu*II and *Eco*RI–*Eco*RV restriction fragments which are diagnostic of normal and mutant γ chain alleles are indicated. Arrowheads indicate the position of oligonucleotides used for the screening of ES cell clones (primers A and B) and for the genotyping of mice (primers C and D). (**B**) Partial nucleotide sequence of the mouse Fbg γ chain gene around the exon 9–exon 10 splice junction. Orange arrows indicate exon–intron boundaries. Amino acids encoded by exon 9 and 10 are shown in green and blue, respectively. The naturally occurring γ chain mRNA splice variant, γ′, is produced as a result of a failure to splice exon 9 and 10 sequences. The C-terminal residues of the γ′ translation product are shown in red. The Gln and Lys residues that participate in factor XIII (transglutaminase)-mediated γ chain crosslinking in the human protein are underlined. Amino acids have been numbered based on the human numbering system since the full amino acid sequence of the mouse γ chain is not known. The nucleotide substitutions introduced into the γ chain gene resulting in a translation termination codon in exon 9 and *Pvu*II site in intron 9 are shown above the normal sequence. In mice homozygous for the mutant γ chain allele, the γΔ5 translation product shown at the bottom is encoded regardless of the mRNA splice pattern. (**C**) Representative Southern blot analysis of *Pvu*II-digested DNA obtained from tail biopsies of wt/wt (lane 1), wt/γΔ5 (lane 2) and γΔ5/γΔ5 mice (lane 3). Positions of restriction fragments corresponding to the endogenous and targeted alleles are indicated.

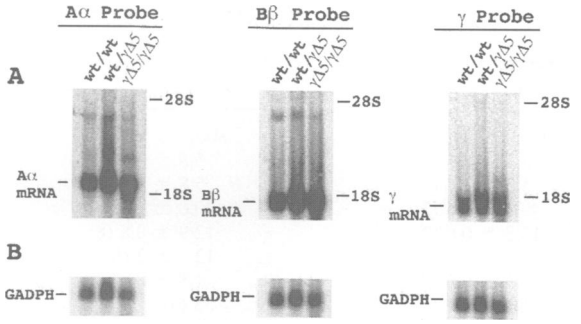

Fig. 2. (**A**) Northern blot analysis of hepatic Fbg mRNA. Fbg Aα (left), Bβ (center) and γ (right) mRNA in total RNA extracts from ~8-week-old wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice. (**B**) The same blots shown in (A) hybridized to a glyceraldehyde 3-phosphate dehydrogenase mRNA-specific probe to control for RNA loading.

γΔ5/γΔ5 mice. Northern blot analysis with chain-specific hybridization probes indicated that both the level and size of each Fbg mRNA species were similar in mice of each genotype (Figure 2).

To determine whether the γ chain mutation had any significant impact on plasma Fbg levels secondary to some unanticipated alteration in translation, modification, assembly, secretion or protein stability, circulating Fbg was examined immunologically. Serially diluted plasma samples from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice were gel-fractionated under reducing conditions and subjected to immunoblot analysis using a polyclonal antiserum recognizing all three mouse Fbg chains. The results indicated that all plasma samples were at least grossly similar with respect to both the amount and size of the individual Fbg polypeptides (Figure 3). The similarity in plasma Fbg concentration in wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice suggested by immunoblot assay was confirmed using a quantitative capture ELISA assay (Table I). Thus, the immunological data argue that the modification made to the γ chain gene had little or no effect on either gene expression or the steady-state levels of circulating Fbg.

Coagulation and general hematological profile in γΔ5/γΔ5 mice

A critical indicator of the structural and functional integrity of the recombinant Fbg expressed in wt/γΔ5 and γΔ5/γΔ5 mice is the functional ability of the Fbg to form a clot in either the plasma milieu or purified preparations. A routine functional assay for plasma Fbg, which is sensitive to both the level and structure of Fbg, is the clotting time after addition of exogenous thrombin (i.e. thrombin-time) (McDonagh *et al.*, 1994). Plasma samples prepared from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice were each found to clot within 12–14 s after the addition of thrombin (Table I). Furthermore, the gross volumes and rigidity of the clots were indistinguishable in samples prepared from animals of each genotype. Similar thrombin-times were also seen in reaction mixtures prepared with 1 mg/ml Fbg purified from wt/wt and γΔ5/γΔ5 mice (data not shown). Importantly, the vast majority of the Fbg purified from γΔ5/γΔ5 mice was able to participate in fibrin polymer formation; like normal mouse Fbg preparations, γΔ5-Fbg was >90% clottable in *in vitro* assays. The γΔ5 mutation also had no secondary impact on the general hematological profile in wt/γΔ5 and γΔ5/γΔ5 mice, including platelet counts, red and white cell counts and hematocrit (Table I).

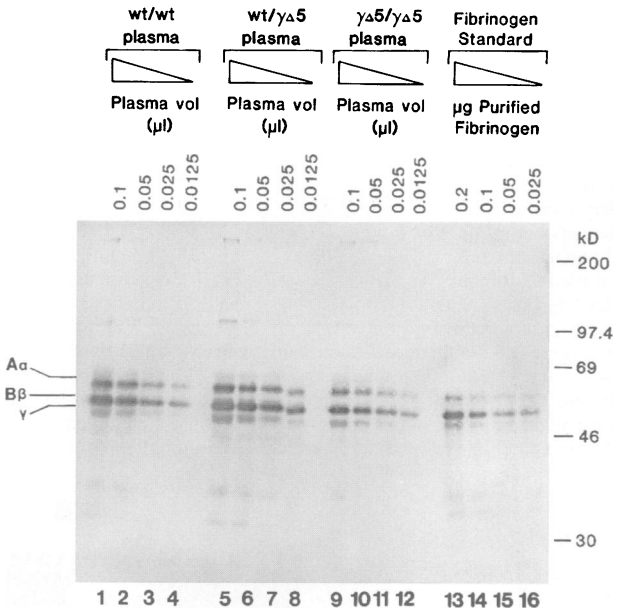

Fig. 3. Immunoblot analysis of Fbg in plasma collected from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice. Serial dilutions of wild-type plasma (lanes 1–4), serial dilutions of wt/γΔ5 plasma (lanes 5–8), serial dilutions of γΔ5/γΔ5 plasma (lanes 9–12) and purified mouse Fbg (lanes 13–16). The positions of the individual Fbg polypeptide chains are indicated on the left.

Crosslinking of the γ chain in fibrin clots formed with γΔ5-fibrinogen

The mutation introduced into the γ chain resulting in γΔ5-Fbg is close to both the donor and acceptor sites for factor XIII (transglutaminase) crosslinking (Figure 1B). To determine whether this would impair γ chain crosslinking, the formation of covalent γ–γ dimers was examined in fibrin clots generated with γΔ5-Fbg. Thrombin added to plasma samples from both wt/wt and γΔ5/γΔ5 mice triggered rapid crosslinking of the γ chains, to form distinct covalently linked γ–γ complexes (Figure 4). The finding that this secondary enzymatic modification of fibrin is not appreciably altered by the γΔ5 mutation provides additional indirect evidence that the general conformation of fibrin(ogen) is likely to be preserved.

Fibrinogen from γΔ5/γΔ5 mice will not support platelet aggregation

To determine whether γΔ5-Fbg also retained the ability to mediate the aggregation of activated platelets, platelet suspensions in autologous plasma were prepared from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice and tested in a standard aggregometer assay. Although control platelet preparations aggregated promptly (Figure 5A) following the addition of the platelet agonist, ADP, platelet suspensions from γΔ5/γΔ5 mice failed to form aggregates (Figure 5E). However, ADP did stimulate platelet shape change (i.e. the rapid reorganization of the platelet cytoskeleton resulting in the conversion of discoid-shaped cells to spherical cells with extended filopodia) as judged by the prompt decrease in light transmission (Figure 5E). Platelet aggregation was also highly impaired in γΔ5/γΔ5 platelet suspensions treated with the potent agonist, collagen (Figure 5F). The seemingly normal platelet aggregation in platelet suspensions from wt/γΔ5 mice is particularly

Table I. Hematological analysis of wild-type and mutant mice

	wt/wt	wt/γΔ5	γΔ5/γΔ5
Platelets ($\times 10^9$/l)	990 ± 30 (3)	920 ± 130 (7)	800 ± 90 (8)
RBC ($\times 10^{12}$/l)	6.6 ± 0.6 (3)	8.1 ± 0.6 (7)	5.8 ± 0.6 (8)
WBC ($\times 10^9$/l)	5.9 ± 0.5 (3)	5.2 ± 2.4 (7)	5.8 ± 1.5 (8)
Hematocrit (%)	33.8 ± 3.1 (3)	40.8 ± 1.6 (7)	40.0 ± 2.3 (8)
Hemoglobin (g/%)	10.9 ± 1.2 (3)	13.3 ± 0.6 (7)	12.9 ± 0.8 (8)
Thrombin time (s)[a]	14 ± 2 (3)	13 ± 1 (7)	12 ± 2 (8)
Plasma Fbg (mg/ml)	1.6 ± 0.2 (3)	1.7 ± 0.2 (3)	1.8 ± 0.1 (3)
Bleeding times (min)	5 ± 2 (12)	6 ± 1 (10)[b]	>25 (5)[c]

Data presented are the mean ± standard deviation with the number of mice analyzed shown in parentheses. Control wild-type mice were NIH Black Swiss except for bleeding time analyses where 129/NIH Black Swiss mice were used.
[a]Plasma clotting times were measured as described in Materials and methods.
[b]Five additional mice in this group were tested, but these failed to stop bleeding when monitored for 16 min (n = 3) and 25 min (n = 2).
[c]The bleeding time test was terminated after 25 min of persistent bleeding.

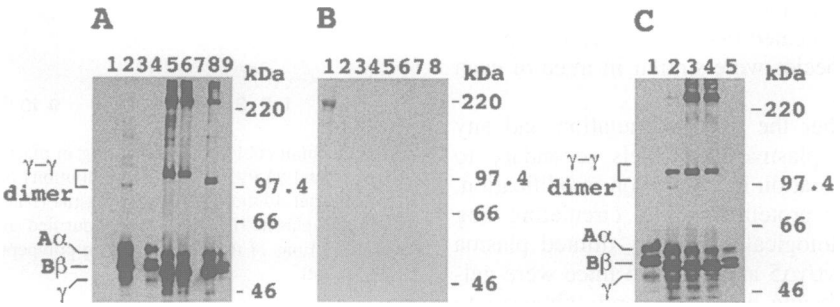

Fig. 4. Covalent crosslinking of the mutant γ chain in γΔ5/γΔ5-plasma clots. (**A**) Immunoblot analysis of fibrin(ogen) detected with a polyclonal rabbit anti-mouse fibrinogen serum. Fbg-deficient plasma (lane 1), wt/wt-plasma (lane 2), no sample loaded (lanes 3 and 7), purified γΔ5-Fbg (lane 4), solubilized clot formed by incubation of thrombin with γΔ5/γΔ5-plasma for either 10 min (lane 5) or 1 h (lane 6), solubilized clot formed by incubation of thrombin with wt/wt-plasma for 1 h (lane 8), solubilized clot formed by incubation of thrombin with wt/wt-plasma for 1 h in the absence of calcium (lane 9). (**B**) Non-immune rabbit serum control blot of samples shown in (A). Molecular mass markers (lane 1), Fbg-deficient plasma (lane 2), wt/wt-plasma (lane 3), purified γΔ5-Fbg (lane 4), solubilized clot formed by incubation of thrombin with γΔ5/γΔ5-plasma for either 10 min (lane 5) or 1 h (lane 6), solubilized clot formed by incubation of thrombin with wt/wt-plasma for 1 h (lane 7), solubilized clot formed by incubation of thrombin with wt/wt-plasma for 1 h in the absence of calcium (lane 8). (**C**) Formation of γ–γ dimer with γΔ5-Fbg as a function of time after thrombin addition. Purified γΔ5-Fbg (lane 1), solubilized clots formed by incubation of thrombin with γΔ5/γΔ5-plasma for 2 min (lane 2), 10 min (lane 3) or 1 h (lane 4), solubilized clot formed by incubation of thrombin with γΔ5/γΔ5-plasma for 1 h in the absence of calcium (lane 5). The positions of the individual Fbg polypeptide chains and γ–γ dimer are shown on the left.

notable in light of the potential dominant-negative effect of Fbg heterodimers (carrying one normal and one mutant γ chain) on fibrinogen-mediated $\alpha_{IIb}\beta_3$ bridging function. These heterodimers (constituting half of all circulating Fbg based on arbitrary γ chain utilization in fibrinogen assembly) would be expected to competitively fill $\alpha_{IIb}\beta_3$ binding sites on platelets, but not provide bridging function. The data suggest that any reduction in either Fbg binding to platelets (see flow cytometry data below) or fibrinogen-mediated bridging function does not markedly reduce aggregation in wt/γΔ5 platelet suspensions under the low shear stress applied in standard aggregometer assays.

The failure of γΔ5/γΔ5 platelets to aggregate was directly related to the γΔ5 mutation in Fbg rather than to an inherent functional deficit in the platelets themselves. Accordingly, the addition of either normal mouse plasma (data not shown) or purified normal mouse Fbg (Figure 5G and H) restored platelet aggregation regardless of the platelet agonist employed. The degree of aggregation achieved in platelet-rich plasma samples prepared from γΔ5/γΔ5 mice was highly dependent on the dosage of purified normal Fbg added to the reaction mixtures (Figure 5G). As little as 25 μg/ml of exogenous normal Fbg supported substantial platelet aggregation (Figure 5G), whereas the mutant γΔ5-Fbg, present in plasma at concentrations nearly two orders of magnitude higher (see above), was unable to support appreciable aggregation. The positive impact of normal Fbg on platelet aggregation did not require that the Fbg be present at the time of platelet activation. Substantial platelet aggregation was also observed in γΔ5/γΔ5 platelet-rich plasma when exogenous normal Fbg was added 1 min after ADP (data not shown).

Reduced binding of γΔ5-fibrinogen to immobilized $\alpha_{IIb}\beta_3$ and activated platelets

Two general hypotheses that could account for the failure of γΔ5-Fbg to support platelet aggregation are: (i) an element is absent that is critical for binding to $\alpha_{IIb}\beta_3$, or (ii) γΔ5-Fbg is readily bound by $\alpha_{IIb}\beta_3$, but this interaction fails to trigger important intracellular events (Shattil *et al.*, 1994) that are essential for aggregation. To distinguish between these possibilities, purified $\alpha_{IIb}\beta_3$ was immobilized in microtiter plate wells and the binding of plasma Fbg from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice was determined immunologically (Figure 6). Plasma samples from mice with a total lack of circulating fibrinogen (Suh *et al.*, 1995) were tested in parallel to control for the specificity of the immunoassay. The amount of Fbg bound in receptor-coated test wells was markedly less in analyses of γΔ5/

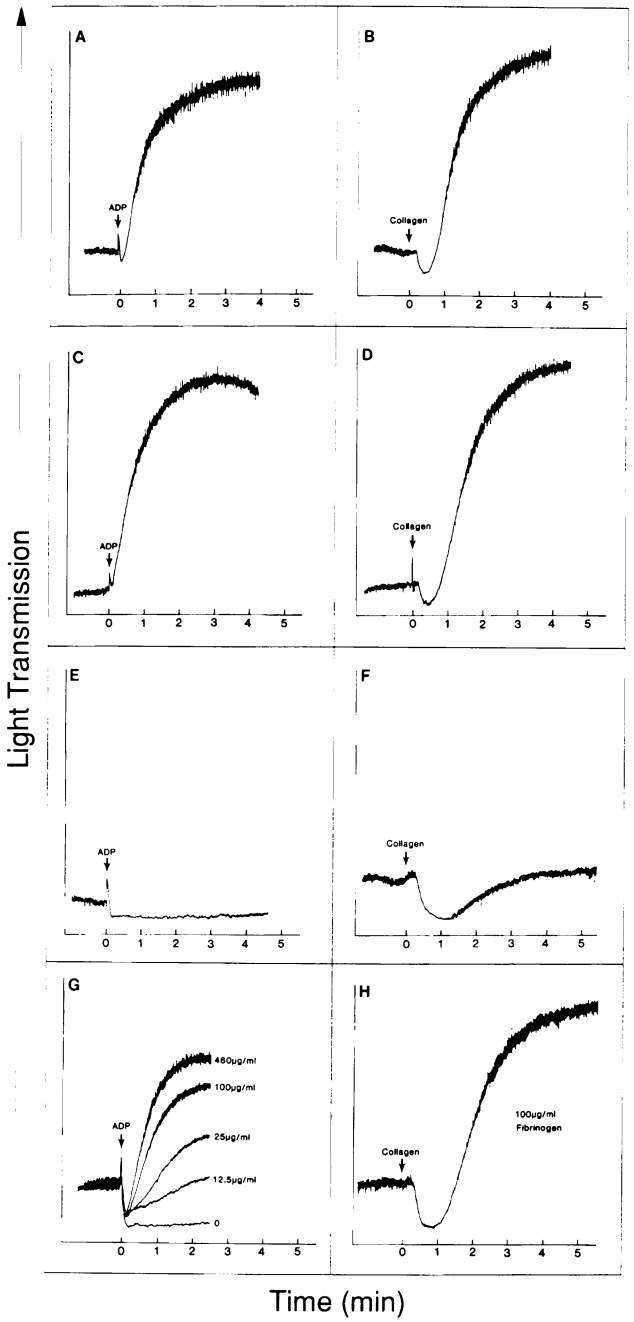

Fig. 5. Aggregation of platelets in plasma suspensions prepared from wt/wt (**A** and **B**), wt/γΔ5 (**C** and **D**) and γΔ5/γΔ5 (**E–H**) mice following the addition of 10 μM ADP (A, C, E and G) or 10 μg/ml collagen (B, D, F and H). Platelet aggregation potential was restored in platelet-rich plasma prepared from γΔ5/γΔ5 mice by the addition of exogenous mouse Fbg (G and H). (G) The aggregation response of γΔ5/γΔ5 platelet suspensions increased in a dose-dependent fashion with added exogenous normal Fbg in the range of 0–460 μg/ml.

γΔ5 plasma than that observed with control plasma collected from wild-type mice (Figure 6). However, a small amount of residual bound γΔ5-Fbg was still detected in these assays even after extensive washing, and this may be due to either non-specific binding or some modest interaction with the four RGD sequences present in the mouse Fbg Aα chain (see Discussion). An intermediate level of Fbg binding was observed with plasma samples collected from heterozygous, wt/γΔ5 mice (Figure 6),

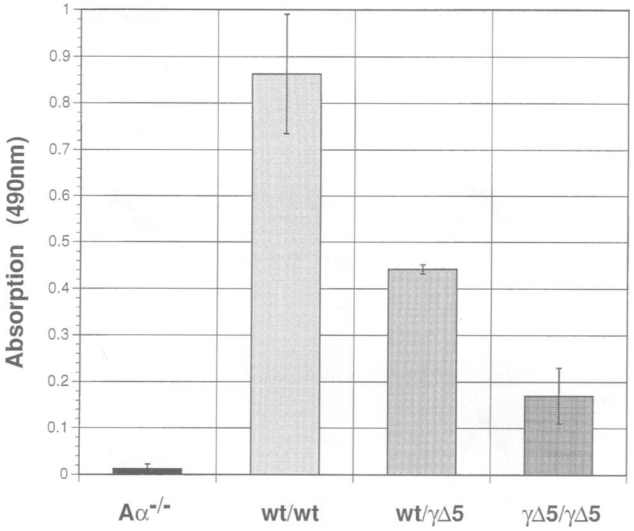

Fig. 6. ELISA assay of plasma Fbg binding to immobilized $\alpha_{IIb}\beta_3$. Plasma samples from Fbg-deficient ($A\alpha^{-/-}$), wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice were analyzed in triplicate and the mean absorbance in the test wells ± SEM (indicated by error bars) is presented.

consistent with the expectation that a substantial portion of the dimeric Fbg in the circulation of these mice would retain at least one fully functional $\alpha_{IIb}\beta_3$ binding motif.

Analyses of Fbg binding to activated platelets using flow cytometry provided further support for the conclusion that the fundamental disorder in γΔ5/γΔ5 mice leading to impaired platelet aggregation is the diminished binding of γΔ5-Fbg by $\alpha_{IIb}\beta_3$. Platelets collected from Fbg-null mice were combined with plasma samples from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice and then activated with 10 μM ADP. Platelet-bound Fbg was then detected using a fluorescein-labeled Fbg antibody (Figure 7). The fluorescence associated with single platelets increased dramatically following the addition of ADP when platelets were suspended in plasma from wt/wt mice. This was apparently due to increased Fbg binding, since no such increase in fluorescence was seen in control analyses of ADP-stimulated platelets suspended in plasma prepared from Fbg-null ($A\alpha^{-/-}$) mice. Importantly, despite the presence of copious amounts of clottable γΔ5-Fbg, platelets suspended in plasma from γΔ5/γΔ5 mice showed almost no change in surface-bound Fbg following platelet activation by ADP (Figure 7). In contrast, substantial Fbg binding was seen with plasma from wt/γΔ5 mice, however the amount of bound Fbg (as indicated by the average fluorescence intensity of single platelets) was significantly less than that observed using plasma samples from wt/wt mice (Figure 7). These results did not reflect a difference in the reactivity of the Fbg antibody with normal and γΔ5-Fbg since the antibody detected both equally well when tested in an indirect ELISA assay (data not shown). Taken together, these data argue that the last five C-terminal residues of the Fbg γ chain, QAGDV, are critical for $\alpha_{IIb}\beta_3$ binding of soluble Fbg and subsequent platelet aggregation.

Platelet-mediated clot retraction is not dependent on the interaction of $\alpha_{IIb}\beta_3$ and the γ chain QAGDV sequence

Clots formed in the presence of platelets retract over a short period of time, a process that may help draw wound

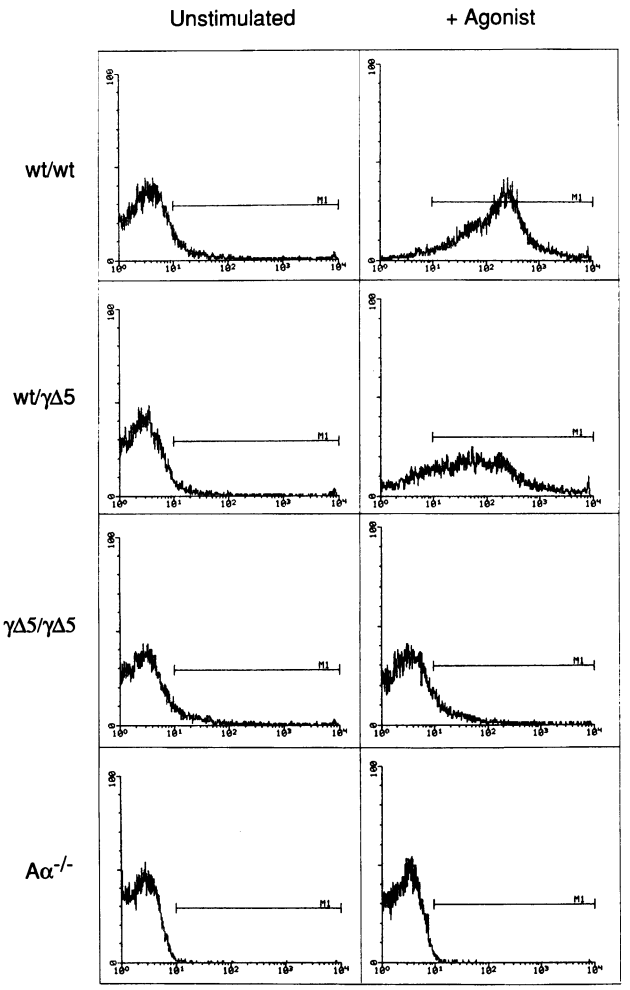

Fig. 7. FACS analysis of the binding of Fbg to resting and activated platelets. Platelets prepared from Fbg-deficient mice were combined with plasma from wt/wt, wt/γΔ5, γΔ5/γΔ5 mice or Fbg-deficient ($A\alpha^{-/-}$) mice and incubated either without agonist (left panels) or with 10 μM ADP (right panels). Bound Fbg was detected using a FITC-conjugated antibody. The platelet number (*y*-axis) is plotted versus platelet fluorescence intensity (*x*-axis). The bar indicates the window of fluorescence intensity above that observed with activated Fbg-null platelet suspensions. The instrument was gated for the analysis of single platelets.

edges together. Although the mechanics are not well understood, retraction requires an interaction between extracellular fibrin and the platelet cytoskeleton via $\alpha_{IIb}\beta_3$ (Lefebvre *et al.*, 1993; Schoenwaelder *et al.*, 1994). Notably, retraction does not occur when clots are formed in the presence of potent $\alpha_{IIb}\beta_3$ inhibitors (Hantgan, 1988; Taylor *et al.*, 1994; Carr *et al.*, 1995), when the platelets incorporated into the clot lack $\alpha_{IIb}\beta_3$ (George *et al.*, 1990; Caen and Rosa, 1995), or when actin filaments are disrupted (Lefebvre *et al.*, 1993). Since the motif recognized by $\alpha_{IIb}\beta_3$ in soluble Fbg was absent in γΔ5-Fbg, we tested whether the loss of this motif also ablated clot retraction (Figure 8). Remarkably, the γΔ5-Fbg supported clot retraction despite the absence of the primary $\alpha_{IIb}\beta_3$ binding motif. Plasma clots formed in the absence of platelets uniformly failed to retract regardless of the genotype of mouse used. Thus, platelet aggregation and clot retraction are both dependent on $\alpha_{IIb}\beta_3$ interaction with fibrin(ogen), but distinct sites appear to participate in each process.

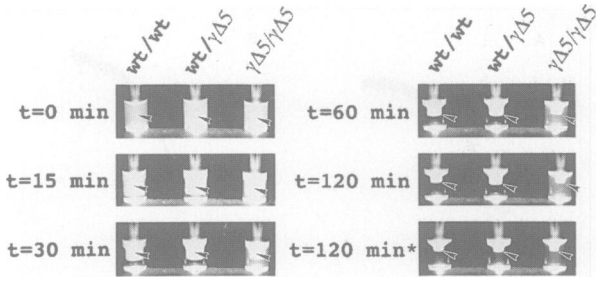

Fig. 8. Platelet-mediated clot retraction is not affected by the γΔ5 mutation. Platelet-rich plasma samples prepared from wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice were clotted by the addition of thrombin and the progress of clot retraction followed over a 120 min incubation period at 37°C. The opaque clots uniformly retracted toward the top surface and are labeled with arrowheads; the bright vertical zones at the top center and the bright horizontal zones at the bottom are due to light reflected off the side and bottom of the glass tubes. Two independent sample sets are shown at the 120 min time point. No clot retraction was observed with any preparation in the absence of platelets (data not shown).

Increased bleeding times in γΔ5/γΔ5 mice

Bleeding events are thought to be initially controlled by the rapid adherence and aggregation of platelets at the site of vascular injury. This concept would argue that γΔ5/γΔ5 mice may be highly ineffective in controlling bleeding events despite seemingly normal clotting function, and no direct restriction on $\alpha_{IIb}\beta_3$ binding to ligands such as vWF and fibronectin. To test this hypothesis, we determined bleeding times in anesthetized wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice, following a standard injury to the nail bed of the fifth digit on the hind leg. Bleeding was brief in wild-type mice (~5 min), whereas, γΔ5/γΔ5 mice, like Fbg-null mice (Suh *et al.*, 1995), persisted in bleeding for as long as they were monitored (>25 min) (see Table I). However, the γΔ5/γΔ5 mice were able to effectively control bleeding at other sites of injury. For example, blood loss was minimal and very brief in these mice following a 1.3 cm full-thickness skin incision, a surgical injury that disrupts relatively small vessels (data not shown). Thus, $\alpha_{IIb}\beta_3$-binding of soluble Fbg appears to be most important in the control of blood loss at sites where larger vessels are compromised.

The control of bleeding in heterozygous mice was of particular interest because of the potential negative impact of circulating Fbg heterodimers on platelet adhesion and aggregation *in vivo*. Consistent with the substantial platelet aggregation seen *in vitro* at low shear stress (see above), most wt/γΔ5 mice were found to rapidly control blood loss; 10 out of 15 wt/γΔ5 mice challenged with a nail bed incision displayed a bleeding time similar to wt/wt mice (Table I). However, five wt/γΔ5 mice showed persistent bleeding similar to γΔ5/γΔ5 mice (Table I). The factors that account for the variability of wt/γΔ5 mice to control bleeding have not been resolved, but this may reflect diminished adhesion/aggregation potential coupled with chance differences between mice in the size of the nail bed vessels ruptured.

Pathological consequences of abolished fibrinogen–platelet binding

Gross and histological analyses of six adult wt/wt, wt/γΔ5 and γΔ5/γΔ5 mice revealed no overt or microscopic abnormalities in any major organ system. Furthermore,

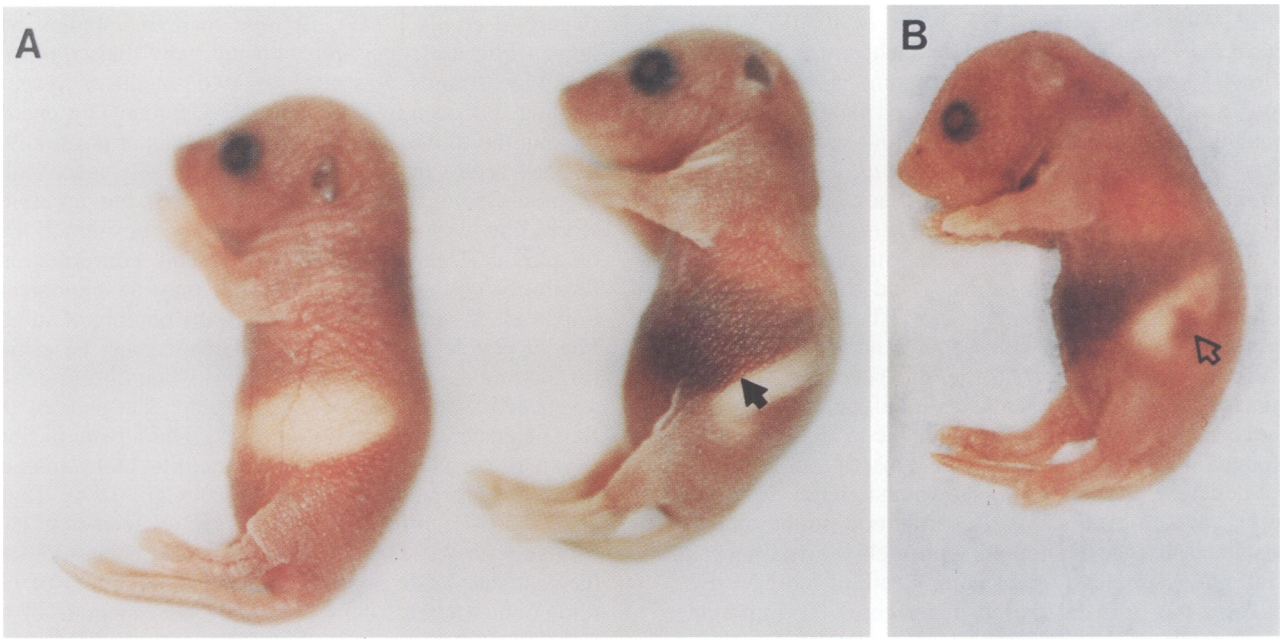

Fig. 9. Spontaneous intra-abdominal bleeding in γΔ5/γΔ5 newborns. (**A**) Two 1-day-old γΔ5/γΔ5 mice littermates. The pup on the right suffered a massive, and ultimately fatal, abdominal bleed that is visible through the body wall (solid arrowhead). (**B**) Another example of a 1-day-old γΔ5/γΔ5 mouse with a severe abdominal bleeding event. The apparent source of bleeding was a ruptured vessel in the stomach wall (open arrowhead).

there were no recorded incidents of overt bleeding, illness or death in >100 mice of each genotype that survived to weaning age and could be monitored for at least 4 months. Both wt/γΔ5 and γΔ5/γΔ5 breeding pairs were capable of reproduction and could generate and sustain multiple litters. The absence of significant bleeding events in pregnant γΔ5/γΔ5 mice is in sharp contrast to Fbg-null females, which uniformly develop fatal intrauterine bleeding events during pregnancy (Suh *et al.*, 1995). However, overt spontaneous bleeding events were noted in a fraction of γΔ5/γΔ5 neonates. Four of 173 (2.3%) γΔ5/γΔ5 neonates closely monitored displayed hemorrhaging into the peritoneal cavity (Figure 9), and these invariably proved fatal. In one case, the source of the free-blood appeared to be ruptured vessels within the distended (milk-engorged) stomach (Figure 9B). Similar intra-abdominal bleeding was documented earlier in Fbg-deficient newborns (Suh *et al.*, 1995), but the penetrance of this phenotype was much higher than that seen in γΔ5/γΔ5 mice.

Discussion

The results presented in this study directly establish that the last five residues of the Fbg γ chain (QAGDV), although not essential for Fbg expression, assembly, secretion or fibrin clot formation, are critical for hemostasis. Mice expressing a form of Fbg lacking these residues displayed a severe defect in platelet aggregation, measured *in vitro*, and were unable to control bleeding following a standard surgical challenge to the nail bed. Furthermore, these animals showed a predisposition to spontaneous fatal bleeding events in the neonatal period, but these events appeared to be rare. The fundamental deficit in γΔ5-Fbg that is likely to lead to sustained bleeding, is the lack of the principal Fbg motif that is bound by the platelet adhesion molecule, $\alpha_{IIb}\beta_3$. As a result, the dimeric Fbg molecule cannot serve as a bridging molecule between $\alpha_{IIb}\beta_3$ moieties on adjacent platelets, platelet aggregation is impaired and hemostatic plug formation *in vivo* is compromised. However, it should be noted that the impact of the γ chain mutation on thrombus formation and control of blood loss may be highly sensitive to the characteristics of the vessel ruptured, including the type of vessel (e.g. arterioles, venules), vessel size and the local shear forces applied by blood flow (see further discussion below).

Although hundreds of human dysfibrinogenemias have been identified (McDonagh *et al.*, 1994), there is no known human correlate of the Fbg mutation described here, whereby clotting function is retained but $\alpha_{IIb}\beta_3$ binding function is specifically lost. However, a comparable disorder, Glanzmann's thrombasthenia (GT), has been known in human patients for almost 80 years (Glanzmann, 1918). GT patients have no Fbg defects that might impede platelet binding, but these individuals specifically lack the platelet receptor $\alpha_{IIb}\beta_3$ (George *et al.*, 1990). As a result, GT patients generally exhibit prolonged bleeding times and severely impaired platelet aggregation and clot retraction, measured *in vitro* (George *et al.*, 1990; Caen and Rosa, 1995). Nevertheless, spontaneous bleeding events are rare in GT patients and most severe hemorrhagic episodes can be traced to either injury or menarche (George *et al.*, 1990; Caen and Rosa, 1995). Given that the γΔ5 mutation only restricts $\alpha_{IIb}\beta_3$ interaction with Fbg, whereas GT restricts or eliminates $\alpha_{IIb}\beta_3$ interaction with all potential ligands [e.g. fibrin(ogen), vWF and fibronectin], one might anticipate that the risk of spontaneous hemorrhage in γΔ5/γΔ5 mice would be relatively low, and significantly lower than that observed in patients with congenital $\alpha_{IIb}\beta_3$ deficiency.

$\alpha_{IIb}\beta_3$-deficient mice are not presently available for direct comparison to γΔ5/γΔ5 animals, but mouse lines with other specific deficits in clotting function and platelet function have been described (Bi *et al.*, 1995; Shivdasani

et al., 1995; Suh *et al.*, 1995; Witke *et al.*, 1995; Bugge *et al.*, 1996). Notably, Fbg-deficient mice have been shown to develop to term, retain some ability to control spontaneous bleeding despite impaired platelet aggregation potential (measured *in vitro* under low shear stress), and generally survive to adulthood (Suh *et al.*, 1995). However, unlike γΔ5/γΔ5 mice, Fbg-null mice lack clotting function, display prolonged bleeding after surgical skin incision (unpublished results), develop catastrophic spontaneous bleeding events as both neonates and adults, and cannot tolerate the challenge of pregnancy. Therefore, a far more profound Fbg disorder than found in γΔ5/γΔ5 mice is compatible with life, but the prospects for long-term survival and reproduction are dim if only fibrin(ogen)-independent mechanisms are available to stem blood loss. Remarkably, recent studies with transcription factor NF-E2-deficient animals have shown that mice develop to term, and can even survive to adulthood, in the total and sustained absence of circulating platelets (Shivdasani *et al.*, 1995). Nevertheless, the bleeding death of most NF-E2$^{-/-}$ mice in the neonatal period underscores the importance of platelets in hemostasis. Clearly, platelet function is sufficient in γΔ5/γΔ5 mice to maintain a normal survival profile in the absence of significant environmental challenges.

Despite the strong affinity of $\alpha_{IIb}\beta_3$ for RGD containing peptides, the results of this study are consistent with the prevailing view that neither of the two RGD sequences found in the human Fbg Aα chain contributes significantly to the binding of $\alpha_{IIb}\beta_3$ to soluble Fbg (Farrell and Thiagarajan, 1994). Indeed, the mouse Fbg Aα chain has four RGD sequences—one in the coiled-coil region (RGDF^{99}, our unpublished results) and three closely spaced in the C-terminal half of the molecule (RGDS^{256}, RGDS^{264}, RGDF^{268}; Murakawa *et al.*, 1993 and our unpublished results). All of these RGD sequences were preserved in γΔ5-Fbg, yet, even in combination, they were not sufficient to support the binding of soluble mouse Fbg to $\alpha_{IIb}\beta_3$. Rather, our results directly show that the RGD-like element in the γ chain, QAGDV, is critical for binding soluble mouse Fbg. However, this short sequence is unlikely to constitute the sole molecular interface between $\alpha_{IIb}\beta_3$ and Fbg. A broader interface or conformational context is implied by the fact that the pentapeptide, QAGDV, is a poor inhibitor of Fbg binding to platelet receptor relative to either the C-terminal dodecapeptide of the γ chain (Kloczewiak *et al.*, 1984) or larger proteolytic fragments containing the C-terminal domain of the γ chain (Savage and Ruggeri, 1991; Kirschbaum *et al.*, 1992). It is interesting to note that the final four residues of the lamprey γ chain contain a true RGD sequence, RGDN (Strong *et al.*, 1985), consistent with the notion that the evolutionary precursor of the mammalian $\alpha_{IIb}\beta_3$ binding motif included RGD.

A still unresolved question is why the four RGD sequences that are present in mouse Fbg fail to mediate $\alpha_{IIb}\beta_3$ binding, whereas the RGD sequences found in other $\alpha_{IIb}\beta_3$ ligands are known to mediate binding (Beacham *et al.*, 1992; Cherny *et al.*, 1993). Two obvious explanations are: (i) the local sequences flanking the RGD elements in Fbg are inappropriate for receptor binding, or (ii) the RGD elements, while suitable for binding, are conformationally inaccessible to receptor in soluble Fbg (Ugarova *et al.*, 1993). Epitope mapping studies with human Fbg are consistent with the view that conformational constraints may exclude RGD-mediated receptor binding. For example, an epitope in human Fbg encompassing the RGD element in the Aα chain at position 95–98, has been shown to be conformationally inaccessible to monoclonal antibody in soluble Fbg, but accessible when Fbg was platelet receptor-bound or immobilized on plastic (Ugarova *et al.*, 1993). This conformational flexibility of the Fbg RGD motifs may be biologically important. While serving no role in the binding of soluble Fbg to platelet receptor, RGD elements may be critical for binding $\alpha_{IIb}\beta_3$, or other integrins, when fibrin(ogen) is associated with a surface. The concept that at least one RGD element can serve as an $\alpha_{IIb}\beta_3$ binding site in fibrin is consistent with the unexpected fact that clot retraction, an $\alpha_{IIb}\beta_3$-dependent event (George *et al.*, 1990; Taylor *et al.*, 1994), proceeds normally in platelet-rich clots formed with γΔ5-Fbg. This model is also consistent with the fact that RGD peptides are potent inhibitors of clot retraction, whereas, the C-terminal dodecapeptide of the γ chain is ineffective in inhibiting clot retraction (Hantgan, 1988). However, we cannot presently distinguish between a model in which a second fibrin-specific binding site for $\alpha_{IIb}\beta_3$ mediates clot retraction, and a model in which a second ligand (e.g. platelet-derived vWF, fibronectin) incorporated into the fibrin matrix mediates clot retraction regardless of the availability of $\alpha_{IIb}\beta_3$ binding sites on fibrin(ogen). Analyses of Fbg mutants lacking both the RGD elements and the γ chain QAGDV motif may be useful in resolving the role of RGD in clot retraction.

The failure of platelet suspensions from γΔ5/γΔ5 mice to aggregate under *in vitro* conditions, devoid of any vessel wall contribution and with minimal shear stress (i.e. the standard aggregometer assay), is unlikely to accurately reflect platelet function and aggregation potential *in vivo*. Platelets are equipped with a variety of adhesion molecules and these can presumably provide some ability to stem blood loss independent of $\alpha_{IIb}\beta_3$–Fbg interactions (Charo *et al.*, 1994). Indeed, the initial binding of platelets to thrombogenic surfaces under high physiological shear stress appears to be primarily dependent on vWF interaction with the platelet receptor, GP Ibα (Savage *et al.*, 1996). Platelet adhesion to immobilized Fbg through $\alpha_{IIb}\beta_3$ and subsequent thrombus formation seem to be most effective at relatively low shear stress. These biological roles of Fbg and vWF in platelet adhesion are probably complementary, rather than redundant, and may provide a mechanism for stable platelet deposition under all conditions of shear stress found in the circulation. It follows from this general hypothesis that the bleeding risk associated with the loss of one class of the platelet adhesion system may vary dramatically between different vascular beds. The minimal bleeding observed in γΔ5/γΔ5 mice following a skin incision and the prolonged bleeding after nail bed injury are consistent with this model.

The γΔ5/γΔ5 mice generated in this study will provide a valuable *in vivo* system for directly examining the role of $\alpha_{IIb}\beta_3$–Fbg interactions in platelet–vessel wall, platelet–platelet, platelet–endothelial cell and platelet–leukocyte adhesion. Of particular interest will be analyses of hemostatic events *in vivo* within selected vessels monitored in real time using videomicroscopy data capture (Frenette

et al., 1995). The role of Fbg in platelet adhesion and thrombus formation could be evaluated in vessels challenged by acute injury (e.g. mechanical vessel rupture or endothelium denudation), or chronic injury (e.g. secondary genetic disorders or infection). Detailed comparative analyses of control, γΔ5/γΔ5 and Fbg-null mice, should ultimately provide further valuable insights into the role of coagulation and Fbg-mediated adhesion events in hemostasis, wound healing, inflammatory response and disease pathobiology.

Materials and methods

Construction of targeting vector and generation of transgenic mice

The Fbg γ chain targeting vector was constructed using a segment of the cloned mouse 129 strain Fbg γ chain gene spanning the region between an *Xba*I site within intron 4 and a *Sal*I site 916 bp downstream of the stop codon in exon 10 (Figure 1). The last five amino acids (QAGDV) in the γ chain were deleted by converting the last codon in exon 9 (CAG) to a translation termination codon (TAG). This mutation (γΔ5) was flagged by two additional nucleotide changes 8 bp downstream of the stop mutation converting CAGCGT to a *Pvu*II site, CAGCTG. These nucleotide substitutions were initially introduced into a 669 bp gene fragment by PCR using a mutagenic primer complementary to the exon 9/intron 9 junction sequence (5′-GGGGATCCAAATAGGTCAGCTGGGATCATGAAGTT-3′) and a downstream primer complementary to an exon 10 sequence (primer D, 5′-ATGGATCCCATTAACTCTGAAAAT-3′). The PCR product was digested with *Bam*HI and the 665 bp fragment was ligated into pBluescript (Stratagene) to generate the recombinant plasmid pBam0.6. The nucleotide sequence of the insert was verified prior to assembly of the final targeting vector (see below).

To introduce the selectable HPRT mini-gene into the 3′ flanking region of the γ chain gene, a unique *Xho*I site was introduced 362 bp downstream of the stop codon in exon 10. This was done by PCR-based mutagenesis using mutagenic γ chain primers (5′-TCTTACCCTCGAGGGGTATGAAACACTCAG-3′ and 5′-CATACCCCTCGAGGGTAAGAACCATGGACG-3′), together with a recombinant plasmid template containing a 0.8 kb *Bam*HI–*Sal*I fragment encompassing a portion of exon 10 and 3′ flanking sequences (Figure 1). The mutated 0.8 kb *Bam*HI–*Sal*I cassette was sequenced to assure the presence of the *Xho*I site (and no unplanned nucleotide substitutions) and then ligated into pBam0.6 to produce pBam-Sal1.5 (insert size 1471 bp). A 2.9 kb *Sal*I cassette containing an HPRT mini-gene (van der Lugt *et al.*, 1991) was inserted into the unique *Xho*I site in pBam-Sal1.5. The targeting vector was completed by introduction of a *Xba*I–*Bam*HI fragment of the γ chain gene spanning intron 4 through exon 9 followed by introduction of a herpes simplex virus-derived thymidine kinase (HSV-tk) mini-gene into the adjacent plasmid polylinker (see Figure 1).

Plasmid-free targeting vector (50 μg) was used to electroporate E14TG2a embryonic stem (ES) cells (Hooper *et al.*, 1987) and stable transfectants were selected as described (Bugge *et al.*, 1995). Clones incorporating the targeting vector by homologous recombination were identified by PCR analysis using a primer complementary to the HPRT cassette (primer A, 5′-CCTGAAGAACGAGATCAGCAGCCTCTGTTC-3′) and a primer complementary to a γ chain 3′-flanking sequence not included in the targeting vector (primer B, 5′-ATACATGGATATTAGCCAGGCAGTAGTGAC-3′). PCR-positive clones were confirmed by Southern blot analysis (Li *et al.*, 1994) of genomic DNA digested with either *Pvu*II or *Eco*RV/*Eco*RI using a 249 bp *Bam*HI–*Xho*I hybrization probe (see Figure 1). The presence of both the stop codon introduced into exon 9 and the *Pvu*II site introduced into intron 9 was established in positive transfectants by cycle sequence analysis using primer C (5′-CCACTCATCACCTAGCAGTTCTG-3′) and primer D (see above) for initial DNA amplification, and a primer complementary to intron 9 (5′-CTTCCAGGCAGATGATT-3′) for sequence analysis.

Chimeric mice generated by injection of ES cells into blastocysts (Bugge *et al.*, 1995) were bred to NIH Black Swiss females (Taconic Farms) to generate mice heterozygous for the targeted gene. These mice were subsequently interbred to generate homozygous (γΔ5/γΔ5) progeny.

Genotyping of mice

The genotypes of mice were determined by either Southern blot analysis of tail biopsy DNA (see above) or analytical *Pvu*II digests of the 1349 bp PCR product generated using the oligonucleotide primers C and D (see above and Figure 1A). In the latter approach, a distinct pattern of *Pvu*II fragments is obtained in wild-type (1011 bp and 338 bp), wt/γΔ5 (1011 bp, 685 bp, 338 bp and 325 bp) and γΔ5/γΔ5 (685 bp, 338 bp and 325 bp) mice.

Fibrinogen expression and hematological parameters

Hematological analysis, platelet aggregation assay, bleeding time analysis and Northern blot analysis were performed as previously described (Suh *et al.*, 1995). For immunoblot analysis of plasma samples, Fbg was detected with a rabbit anti-mouse Fbg serum. Bound primary antibody was detected either with a phosphatase-conjugated donkey anti-rabbit IgG antibody (Sigma) and the 5-bromo-4-chloro-3-indoyl-phosphate BCIP/nitroblue tetrazolium (NBT) (Sigma) staining system or a peroxidase-conjugated donkey anti-rabbit IgG (Amersham) and the ECL (Amersham) chemiluminescence system. Fbg was measured quantitatively in mouse plasma by a Fbg-specific ELISA (Asserachrom-Fibrinogen kit, Diagnostica Stago) using a purified mouse Fbg standard.

Clot retraction

Platelet-rich plasma was prepared as described (Suh *et al.*, 1995) with EDTA used as anticoagulant. Platelet suspensions in 450 μl plasma (10^8 platelets/ml) were recalcified with 50 μl 50 mM $CaCl_2$, and clotted by addition of 1 U of bovine thrombin (Pacific Hemostasis). The samples were incubated at 37°C and the extent of clot retraction was recorded photographically over a 2 h period.

Purification of plasma fibrinogen

Fbg was purified from citrated plasma by serial ammonium sulfate precipitations. Briefly, plasma was adjusted to 10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 100 mM NaCl, 200 000 U/ml aprotinin (Calbiochem) and plasma proteins were precipitated by addition of ammonium sulfate to 25% of saturation. The protein precipitate was collected by centrifugation at 7000 *g* for 15 min at room temperature and washed twice with precipitation buffer. The precipitate was resolubilized in 10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 100 mM NaCl, 200 000 U/ml aprotinin and the ammonium sulfate precipitation repeated. Following two more washes in precipitation buffer, the Fbg was resuspended in 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 137 mM NaCl and dialyzed exhaustively against resuspension buffer. Fbg concentration was established spectophotometrically (Kuyas *et al.*, 1990).

Crosslinking of plasma fibrinogen

The covalent crosslinking of the mutant Fbg γ chain was evaluated in whole plasma clots formed *in vitro*. Citrated plasma was combined with one-tenth volume of 125 mM Tris–HCl, pH 7.4, 100 mM cysteine, 100 mM $CaCl_2$, 60 U/ml bovine thrombin and incubated at 37°C. Under these conditions, clot formation occurred in <20 s. For samples in which factor XIII transglutaminase activity was inhibited by chelation of divalent cations, the plasma clots were formed in the absence of calcium and in the presence of 10 mM EDTA. Soluble plasma proteins were removed by exhaustively washing the clot in 10 mM Tris–HCl, pH 7.4, 20 mM EDTA, 137 mM NaCl. The clot was solublized by addition of 100 mM Tris–HCl, pH 6.8, containing 8 M urea, 2.0% SDS and 200 mM β-mercaptoethanol and incubating at 75°C. Samples were then fractionated by SDS–PAGE and analyzed by immunoblotting as described above.

Binding of fibrinogen to immobilized $\alpha_{IIb}\beta_3$

Maxisorb plates (Nunc) were coated with 1 μg of soluble human $\alpha_{IIb}\beta_3$ (Enzyme Research Laboratories, Inc.) in 100 μl of 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 7 mM NaN_3 (coat buffer) and then blocked with coat buffer containing 3% bovine serum albumin (BSA). Mouse plasma samples were diluted 1:400 in 25 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 0.5% (v/v) Tween-20 and 50 μl aliquots were incubated in microtiter wells for 2 h at room temperature. The wells were washed in the dilution buffer and then incubated with 100 μl rabbit anti-mouse Fbg serum (pre-absorbed with Fbg-deficient mouse plasma) diluted 1:2000 in dilution buffer containing 0.5% BSA. Bound immunoglobulin was detected using peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham) and OPD chromogenic substrate (American Diagnostica).

Flow cytometric analysis

Fbg binding to activated platelets was assayed by FACS analysis using a FITC-conjugated rabbit anti-human Fbg IgG (Dako). Briefly, 5 μl of platelet-rich plasma prepared from Fbg-null mice (Suh *et al.*, 1995) were

combined with 5 μl of test plasma, 5 μl antibody (1.1 mg/ml) and 39 μl 10 mM HEPES, pH 7.4, containing 145 mM NaCl and 5 mM KCl. Platelets were activated by addition of 6 μl of 100 mM ADP, incubated at room temperature for 20 min without stirring, and then fixed by the addition of 0.5 ml 0.2% (v/v) formaldehyde in phosphate buffered saline. Fixed platelet suspensions were analyzed using a FACS-Scan flow cytometer (Becton-Dickinson) with the instrument gated for the detection of single platelets. To show that the Fbg antibody used in the flow studies bound similarly to plasma Fbg from control and γΔ5/γΔ5 mice, an indirect Fbg ELISA was performed. Briefly, microtiter plates (Titertek) were coated with 200 μl wt/wt or γΔ5/γΔ5 mouse plasma serially diluted in 100 mM $NaHCO_3$. The wells were washed in 13 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.75% (v/v) Tween-20, 5 mM NaN_3 and then blocked in the same buffer containing 1% BSA. FITC-conjugated rabbit anti-human Fbg IgG was diluted 1:2000 in blocking buffer and 200 μl was added to each test well. Following a 2 h incubation at room temperature, the wells were washed and bound primary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham) as described in the section above.

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